Role of DDAH/ADMA pathway in TGF-β1-mediated activation of hepatic stellate cells

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Abstract. Asymmetric dimethylarginine (ADMA) is catalyzed by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) in humans, and the role for ADMA has been associated with hepatic fibrogenesis. Transforming growth factor-β (TGF-β) has been shown to mediate the myofibroblastic transformation of quiescent hepatic stellate cells (HSCs), a pivotal step in liver fibrogenesis. However, the underlying molecular mechanisms are not well understood. Accumulation of ADMA due to low activity of DDAH has been reported to be associated with liver damage and hepatic fibrosis. In this study, the role of the DDAH/ADMA pathway in the TGF-β1-induced HSC activation was assessed. Freshly harvested primary HSCs from rat liver were used in this study. It was demonstrated that TGF-β1 treatment significantly suppressed the DDAH protein expression and activity, and increased levels of ADMA in the culture medium of rat primary HSCs. Notably, the TGF-β1-mediated effects on DDAH/ADMA were significantly abrogated by the p38 mitogen activated protein kinase specific inhibitor, SB203580. Furthermore, it was demonstrated that excessive ADMA led to an increase in the number of TGF-β1-positive HSCs and induced the expression of α-smooth muscle actin and collagen type I in rat primary HSCs. In addition, rat primary HSCs exposed to excessive ADMA showed a significant increase in the expressions of α-SMA and collagen type I. Finally, it was revealed that ADMA treatment promoted the proliferation of rat primary HSCs. In conclusion, the results obtained from the study suggest a potentially novel role for the ADMA/DDAH1 signaling pathway in TGF-β1-induced HSC activation, and along with the studies of others, suppression of the ADMA/DDAH1 pathway may be an alterative approach for the treatment of liver fibrosis.

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Introduction

Chronic liver injury generally leads to hepatic fibrosis and may subsequently progress to cirrhosis, a serious liver disease that may eventually result in the development of hepatocellular carcinoma (HCC) (1-3). Numerous studies have demonstrated that the activation of hepatic stellate cells (HSCs), which store fat in the liver, represent a pivotal event in the initiation of hepatic fibrogenesis, during which quiescent HSCs specialized for retinoid-storage are transformed to contractile cells termed myofibroblast as a result of liver damage (1). Activated HSCs or myofibroblasts are characterized by positive expression of α-smooth muscle actin (α-SMA) and by secretion of the extracellular matrix (ECM) components such as collagen type I and collagen type II, which are mainly responsible for the formation of scar tissue and the development of cirrhosis (1). This phenotype shift of HSCs is also accompanied by an increase in cell proliferation and contractility of activated HSCs. Transforming growth factor-β (TGF-β) has been shown to mediate myofibroblastic transformation of quiescent HSCs and accumulation of ECM in response to liver injury (4-8). The TGF-β family of cytokines possesses three isoforms, TGF-β1, TGF-β2, and TGF-β3. These regulate various cellular processes including cell proliferation, apoptosis, differentiation, and migration through a number of signaling pathways (4,9). However, the molecular mechanisms involved in the activation of HSCs are not well understood.

Nitric oxide (NO) is an important cell signaling molecule produced from oxidation of L-arginine, a step that is enzymatically catalyzed by NO synthase (NOS). Studies have shown that the activity of NOS is competitively inhibited by asymmetric dimethylarginine (ADMA), a naturally occurring analogue of L-arginine in humans. In humans, ADMA is catabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH); abnormal down-regulation of DDAH or inhibition of its activity could result in accumulation of ADMA in the plasma. DDAH has two isoforms: DDAH1, a major isoform in the liver, and DDAH2 which is mainly expressed in the endothelial cells (10). In a recent study, over-expression of DDAH was shown to prevent renal fibrosis via suppression of ADMA, a well-known inhibitor of NOS, and therefore reduction of ADMA would lead to an increase in the production of NO (11,12). Elevated plasma levels of ADMA were also found to be strongly associated with the degree of liver damage and hepatic fibrosis (13-15). Interestingly, rat primary HSCs exposed to excessive ADMA displayed strong induction of α-SMA and collagen type I, which suggests that the ADMA/DDAH pathway could be involved in hepatic fibrogenesis in response to liver injury (16-18).

In the present study, we investigated the role of the ADMA/DDAH pathway in the TGF-β1-induced activation of freshly isolated rat primary HSCs. We furher examined if the MAPK pathway could participient in the TGF-β1-associated effects on DDAH/ADMA by using specific inhibiors for three main subgroups in the MAPK superfamily [p38 kinases (p38), extracellular signal regulated kinases (ERK), and c-Jun N-terminal kinases (JNK)]. Our findings may help understand the molecular mechanisms involved in activation of HSCs, and help identify target molecules for the development of preventive and therapeutic approaches to liver fibrosis.

Materials and methods

Reagents and materials. Rat anti-α-SMA monoclonal antibody and the enzyme linked immunosorbent assay (ELISA) kit for collagen type I were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany); DMEM cell culture medium was from Gibco (Cambridge, UK); Mitogen-activated protein kinase (MAPK) specific inhibitors SB203580, PD98059, and SP600125 were purchased from Beyotime Biotechnology (Shanghai, China); Anti-DDAH1 and anti-TGF-β1 antibodies were purchased from Abcam (Grand Island, NY, USA).

Preparation, culture, and verification of the rat primary HSCs. Male Sprague-Dawley (SD) rats (body weight: 350 to 400 g) were obtained from the Shanghai SLAC Laboratory Animal Inc. (Shanghai, China). Fresh liver tissues of the SD rats were used for isolation of primary HSCs. Prior to the experiments, all SD rats were maintained in the Animal Center of Hunan Province and handled according to the protocols that were approved by the Animal Care and Ethics Committee at the Animal Center of Hunan Province [no. SCXK (Xing) 2011-0003]. Rat primary HSCs were prepared and cultured as described elsewhere (19-21). Briefly, primary HSCs were isolated by digestion of the freshly harvested rat liver tissues with *in situ* perfusion of pronase and collagenase, followed by a single-step density Nycodenz gradient centrifugation. During the centrifugation, HSCs cells were separated from other hepatic cells due to the high lipid content of HSCs.

Trypan Blue staining was used to assess the viability of the freshly prepared HSCs. In brief, 100 μl of the HSCs suspension were mixed with the equal value of 0.4% typan blue solution, and then 10 μl of the resulting suspension were dropped on a slide with a cell counting chamber, which was followed by calculating the numbers of viable and nonviable HSCs under an optical microscope (Olympus Corporation, Tokyo, Japan). Desmin immunocytochemistry was utilized to examine the purity of the isolated HSCs. Briefly, HSCs were fixed, permeabilized, and incubated overnight in primary antibodies. Following three washes with PBS, bound primary antibodies were detected with secondary antibody. HSCs were visualized and characterized by microscopy using a microscope (Olympus Corporation). Next, Image-Pro Plus software 6.0 (Media Cybernetics, Silver Spring, MD, USA) was used for analysis of the images, and the average optical density (AOD) value was calculated to represent the expression levels of desmin. Freshly prepared HSCs were grown in DMEM cell culture medium supplemented with 20% fetal bovine serum (FBS) for 24 h, and the culture medium was replaced with DMEM containing reduced concentration FBS (0.5%), cultured for another 24 h, and followed by exposure to different treatments.

Treatments of the rat primary HSCs. The HSCs were subsequently divided into different groups as per the experimental protocol. To determine the effect of TGF-β1 on DDAH/ADMA, HSCs were treated with different concentrations of TGF-β1 (0, 1, 2.5, and 5 ng/ml) for 48 h. HSCs were harvested for subsequent analysis of the protein expression of DDAH1 and ADMA, and the activity of DDAH. In the ADMA treatment group, HSCs were incubated with ADMA (0, 1.0, 2.5, 5.0 μM) for 48 h, cell culture medium and HSCs were collected for subsequent measurement. In some experiments, we included specific inhibitors of the mitogen-activated protein kinase (MAPK) superfamily: p38 kinases (p38) extracellular signal regulated kinases (ERK), and c-Jun N-terminal kinases (JNK). Three inhibitors specific for p38 (SB203580), ERK (PD98059), and JNK (SP600125) were selected, and HSC cells were pretreated with 2 μM of SB203580, PD98059, and SP600125 for 30 min, prior to exposure to 5 ng/ml of TGF-β1. 48 h post treatment, HSCs and cell culture were harvested for subsequent analyses.

Immunohistochemical examination for α-SMA positive HSCs. HSCs with positive expression of α-SMA were assessed in the different experimental groups after immunohistochemical staining using anti-α-SMA monoclonal antibodies. α-SMA-positive HSCs were defined as HSCs with light or dark brown particles localized in the cell membrane and/or cytoplasm. The staining intensity was assessed using Image-ProPlus image processing software and quantified by calculating the AOD.

ELISA for collagen type I expression. ELISA for type I collagen was performed according to the manufacturer's instructions (Sigma-Aldrich; Merck KGaA). In brief, the HSCs culture medium was collected from the experimental groups, diluted with PBS, and subsequently incubated with rabbit anti-rat anti-type I collagen antiserum (1:2,000) and goat anti-rabbit IgG HRP (1:1,000). 2 ml/l of H_2SO_4 was used to terminate the reaction. Fresh serum-free medium was used as negative control. The concentrations of type I collagen in the cell culture medium were calculated by the absorbance obtained at wavelength of 490 nm on a spectrophotometer.

Measurement of ADMA in the cell culture medium and DDAH activity assay. Levels of ADMA in the cell culture medium were measured with high performance liquid chromatography (HPLC), as described elsewhere (22). The DDAH activity assay was carried out as reported previously (22). In brief, ADMA was added to HSCs lysates at a final concentration of 500 μM, followed by incubation at 37˚C for 2 h. 30% of sulfurosalicylic acid, which is able to inactivate DDAH, was used to terminate the reaction. The remaining ADMA was measured by HPLC, and the reduction of ADMA amount reflected the DDAH activity. DDAH activity in the various experimental groups was expressed relative to that of the normal control group.

Western blotting for protein expression of DDAH1 and TGF-β1. Western blot analysis was performed to examine the protein levels of DDAH1 and TGF-β1. In brief, total proteins extracted from the HSCs in various experimental groups were separated on 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were subsequently transferred onto ImmunBlot PVDF membranes and incubated with anti-DDAH1 (1:2,000) and anti-TGF-β1 (1:5,000) primary antibodies, respectively, at room temperature for 2 h. The resulting bands were visualized using ECL and analyzed on an Image J analyzer imaging system. The intensity of DDAH1 and TGF-β1 bands was normalized to that of β-actin as an internal control.

Cell proliferation assay. MTT assay was used to assess cell proliferation in the control group, and three experimental groups: TGF-β1, ADMA, and SB203580. The HSCs were exposed to 5.0 ng/ml TGF-β1, 5.0 μM ADMA, or 3.0 μM SB203580 for different durations of time (0, 12, 24, 48, 72, 96 h). 10 μl of MTT working solution (5 mg/ml) was added to each well, incubated at 37˚C for 4 h, and mixed with 100 μl of DMSO. Absorbance (A) was obtained on a microplate reader at a wavelength of 490 nm.

Statistical analysis. All experiments in this study were performed for a minimum of three times and all data were presented as mean values with standard error (SE) (mean ± SE). Statistical analysis was performed with IBM SPSS Statistics version 17.0 from SPSS, Inc. (Chicago, IL, USA). Multi-factor analysis of variance (ANOVA) was used to detect significant differences. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Viability and purity of the freshly prepared rat primary HSCs. We first isolated primary HSCs from the liver tissues of SD rats. The yield was approximately 7×10^7 cells per rat. The viability of freshly prepared HSCs was determined by Trypan Blue staining and was found to be more than 92% (data not shown). The purity of the isolated HSCs was examined using immunohistochemical staining for Desmin. Results were analyzed under phase-contract microscopy, in combination with analysis by ultraviolet-excited fluorescence microscopy. The purity was greater than 90% (data not shown). The high viability and purity of the extracted rat primary rat HSCs met the requirement for the proposed experiments in this study.

Effects of excessive TGF-β1 on the expression and activity of DDAH, and levels of ADMA. With the freshly isolated rat primary HSCs, we first determined effects of TGF-β1 on the protein expression and activity of DDAH in the HSCs, as well as levels of ADMA in the culture medium. In this study, the isoform DDAH1 rather than DDAH2 was selected. On exposure to various concentrations of TGF-β1 (1, 2.5 and 5 ng/ml), both DDAH1 protein expression and activity of the HSC cells were significantly reduced as compared to that in controls ($P<0.05$). Moreover, the effect was highly dose-dependent (Fig. 1A and B). TGF-β1 concentrations of 2.5 and 5 ng/ml caused a significant increase in ADMA in the cell culture medium as compared to that in controls ($P<0.05$; Fig. 1C).

Role of p38 MAPK pathway in the TGF-β1-associated effects on DDAH/ADMA. We next explored the potential involvement of the MAPK molecular pathway in the TGF-β1-associated effects on DDAH/ADMA, and included three inhibitors specific for p38 (SB203580), ERK (PD98059), and JNK (SP600125) in the subsequent experiments. Freshly prepared rat primary HSCs were treated with the p38 MAPK specific inhibitor SB203580 or vehicle only as control. We also included an ERK inhibitor (PD98059) and JNK inhibitor (SP600125) to determine if p38 MAPK could be specific to the TGF-β1-induced alteration on DDAH/ADMA, and also to exclude the potential contribution of ERK and JNK in the observed effects. p38MAPK specific inhibitor inhibited the TGF-β1-induced effects on DDAH/ADMA, while protein expression of DDAH1 and activity of DDAH increased, and protein levels of ADMA in the cell culture medium decreased ($P<0.05$ vs. TGF-β1 alone). In contrast, neither ERK inhibitor PD98059 nor JNK inhibitor SP600125 displayed any effect on the TGF-β1-associated effects on DDAH/ADMA (Fig. 2). We then examined the effect of different doses of the p38MAPK specific inhibitor (0, 1, 2 and 3 μM) on TGF-β1-induced effects on DDAH/ADMA. The results showed that the effects on the protein levels of DDAH1 and ADMA, and on the activity of DDAH were dose-dependent (Fig. 3).

Effects of excessive ADMA on levels of α-SMA and collagen type I. We next asked if increased levels of ADMA induced by TGF-β1 could alter the expressions of fibrotic markers α-SMA and collagen type I. We found that the cell number of HSCs positive for α-SMA increased significantly in response to excessive ADMA treatment at different concentrations (0, 1, 2.5 and 5 μM) as compared to that in the controls, and that the effect was dose-dependent ($P<0.05$; Fig. 4A-C). Rat primary HSCs exposed to ADMA (2.5 and 5 μM) showed significant enhancement in the production of collagen type I vs. control cells without ADMA treatment ($P<0.05$; Fig. 4D). Moreover, excessive ADMA at the indicated concentrations (1, 2.5 and 5 μM) significantly increased the protein expression of collagen type I in comparison with control HSCs without exposure to ADMA ($P<0.05$; Fig. 4E and F).

Effects of ADMA and the p38 MAPK specific inhibitor on the proliferation of HSCs. Since cell proliferation is generally promoted in the activated HSCs, we further evaluated the role for the DDAH/ADMA pathway on the hepatic fibrogenesis and conducted comparative studies to assess the effects

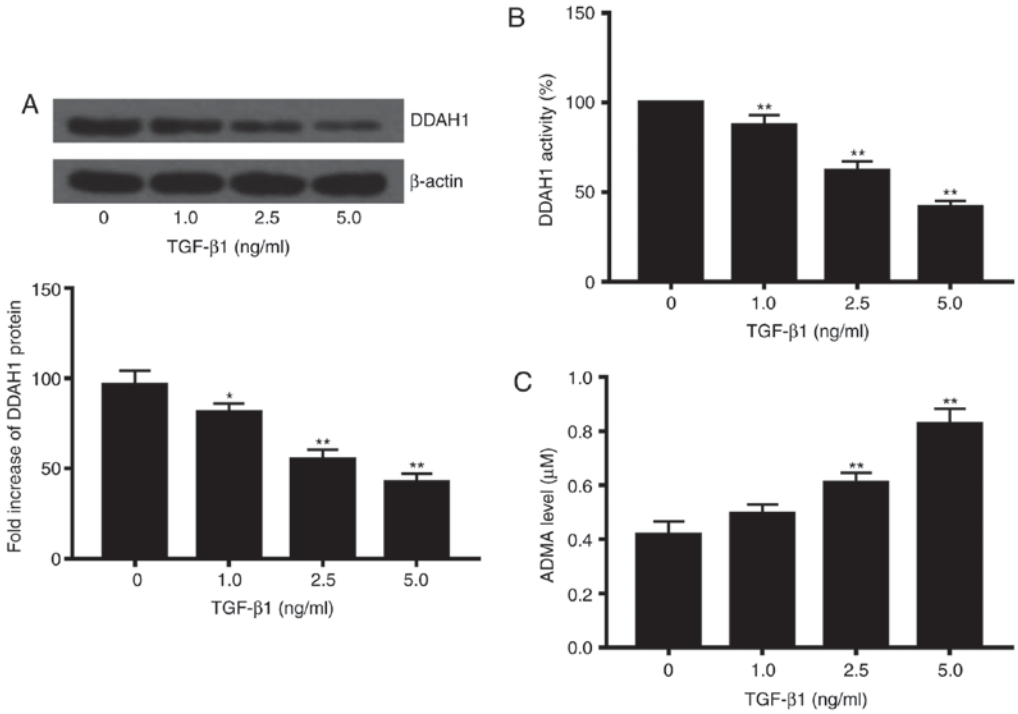

Figure 1. Effects of excessive transforming growth factor (TGF)-β1 on the protein expression and activity of dimethylaminohydrolase (DDAH), and levels of asymmetric dimethylarginine (ADMA) in cell culture medium. Rat primary hepatic stellate cells (HSCs) were treated with TGF-β1 (0, 1.0, 2.5 and 5.0 ng/ml) for 48 h. DDAH1 protein expression was measured by western blot analysis and the activity of DDAH1 was measured as described in Materials and Methods. Levels of ADMA in the cell culture medium were quantified by HPLC. $^{*}P<0.05$, $^{**}P<0.01$ vs. control. (A) Effect of TGF-β1 on protein expression of DDAH1; (B) Effect of TGF-β1 on the activity of DDAH1. The activity of DDAH1 in control HSCs without exposure to TGF-β1 was set as 100%; (C) Effects of TGF-β on protein expression of ADMA.

of excessive ADMA, TGF-β1, and the p38 MAPK specific inhibitor on the proliferation of HSCs. TGF-β1 showed the greatest increase in the proliferation of HSCs, followed by the ADMA treatment in contrast to controls, whereas the p38 MAPK displayed a significant inhibitory effect on the growth of HSCs as compared to that of control HSCs (P<0.01; Fig. 5).

Discussion

The molecular mechanisms whereby TGF-β induces the activation of HSCs are not well understood. The key novel findings from this study are as follows: i) TGF-β1 significantly suppressed the DDAH protein expression and activity, and increased levels of ADMA in rat primary HSCs (Fig. 1); ii) the TGF-β1-mediated effects on DDAH/ADMA were significantly abrogated by the p38 MAPK specific inhibitor SB203580, but not the ERK- and JNK-specific inhibitors (Figs. 2 and 3); and iii) Enhancement of ADMA significantly induced expression of α-SMA and type I collagen in the rat primary HSCs (Fig. 4), and promoted cell proliferation of HSCs as compared to that in controls (Fig. 5).

It has been well-documented that the TGF-β family members including TGF-β1, TGF-β2, and TGF-β3 in mammals are major mediators of fibrosis in response to tissue injury of the liver and kidney through the TGF-β-mediated signaling pathways (5,9). In the present study, we demonstrated that TGF-β1 suppressed the protein expression and activity of DDAH, and in turn led to an accumulation of ADMA in the culture medium of rat primary HSCs, and that the p38 MAPK pathway participated in the TGF-β1-associated effects on DDAH/ADMA. The findings, to our knowledge, have not been reported previously and may represent an important advance in our understanding of the roles of TGF-β1 and ADMA in the pathogenesis of hepatic and renal fibrosis. It has been reported that the regulation of DDAH activity by TGF-β1 comprises of both inhibition of translation activity and that of translation level protein expression (5,9). In this study, it is likely that reduced protein expression of DDAH1 by TGF-β1 led to a decrease in its activity. Of note, the above TGF-β1-mediated effects on DDAH/ADMA were abrogated by the presence of p38 MAPK specific inhibitor, but not by the inhibitors of JNK and ERK, which indicates an involvement of p38 MAPK pathway rather than JNK-MAPK and ERK-MAPK pathways in this effect.

The activation of HSCs is well recognized as a central event in hepatic fibrosis, which involves a range of signaling pathways. A number of studies have shown that ADMA, an endogenous molecule (23), is associated with the severity and progression of liver damage (15,24-27). In a study by Li *et al* (28), it was important to note that excessive ADMA significantly induced mRNA expression of α-SMA, increased α-SMA-positive cells ratio, and synthesis of type I collagen in dose- and time-dependent manners in HSCs through, at least in part, the ROS-NF-κB molecular pathway, which suggests that ADMA is involved in the activation of HSCs as a potentially novel mediator of transformation of HSCs (28). Besides its potential role in liver fibrosis, ADMA has also been implicated in renal fibrosis (29,30) and in tubuleinterstitial ischaemia on the early stage in diabetic nephropathy (DN) (31). With a rat model of DN, Shibata *et al* (31) found that reduction of AMDA

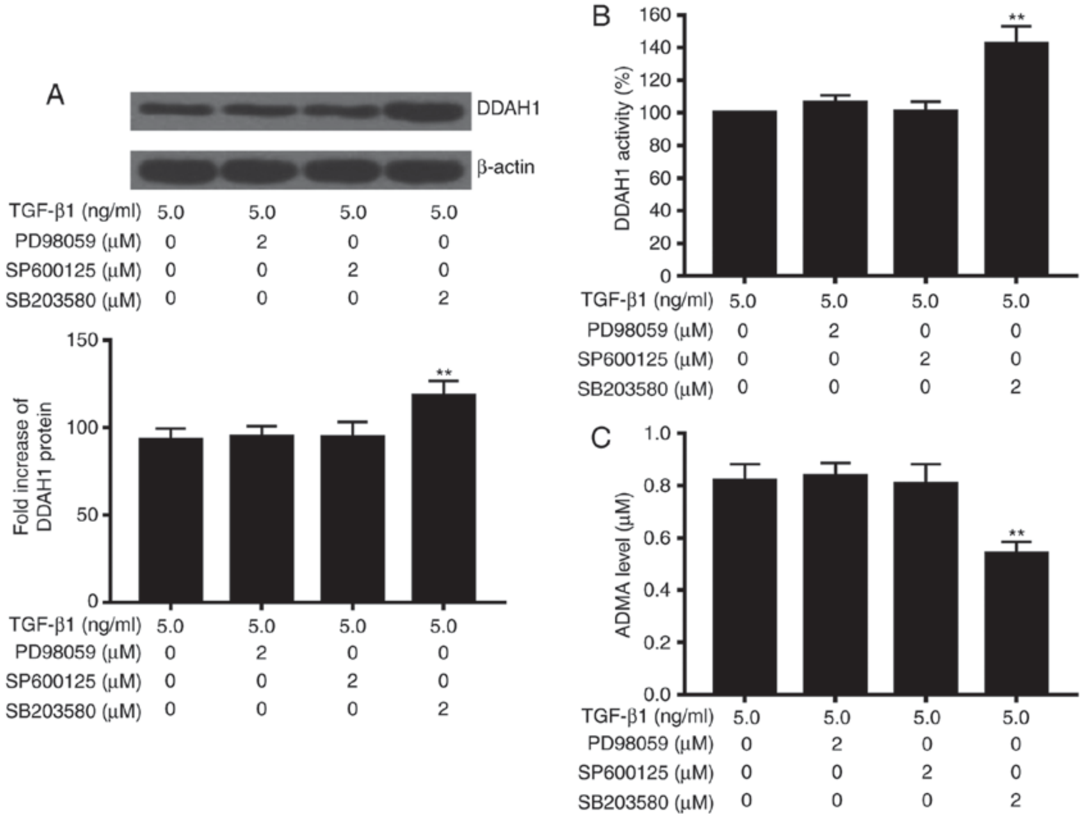

Figure 2. Effect of p38 MAPK specific inhibitor (SB203580) on transforming growth factor (TGF)-β1-mediated effect on asymmetric dimethylarginine (ADMA) and dimethylaminohydrolase (DDAH). Rat primary hepatic stellate cells (HSCs) were treated with TGF-β1 (5.0 ng/ml) alone or in combination with p38 MAPK specific inhibitor SB203580, ERK inhibitor PD98059, or JNK inhibitor SP600125. Protein levels of DDAH1 and ADMA, and activity of DDAH1 were assayed as described in Materials and Methods. (A) Effect of p38 MAPK specific inhibitor SB203580 on TGF-β-mediated action on protein expression of DDAH1; (B) DDAH1 activity; (C) Inhibitory effect of p38 MAPK specific inhibitor SB203580 on TGF-β-mediated effect on ADMA levels. **P<0.01 vs. control.

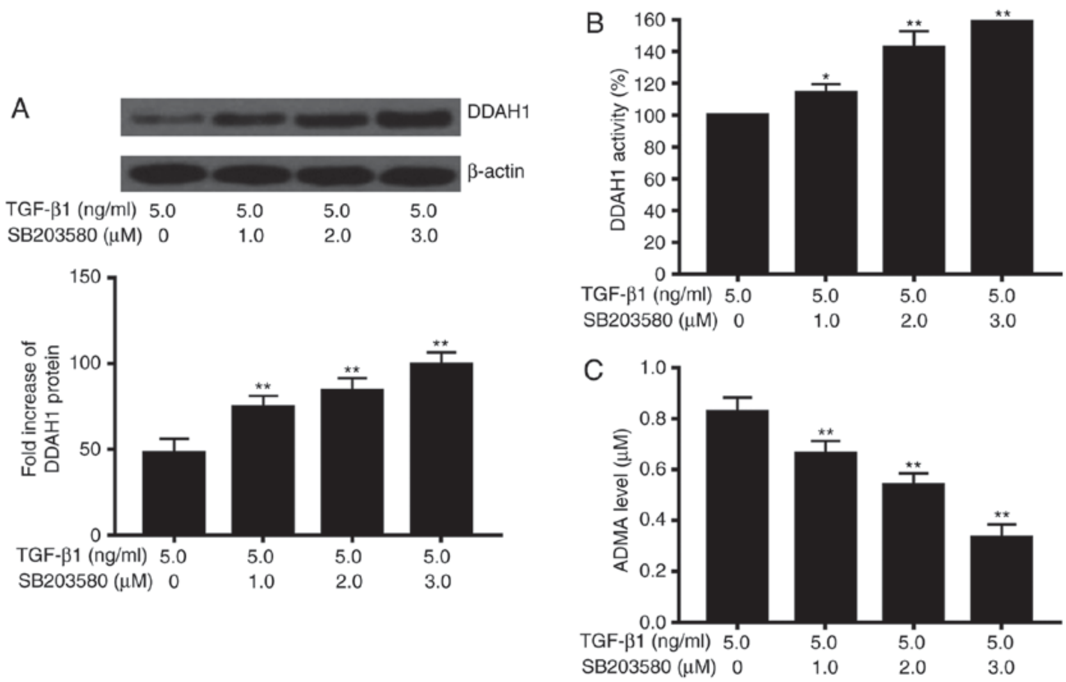

Figure 3. Dose-dependent effect of P38 MAPK specific inhibitor SB203580 on transforming growth factor (TGF)-β1-mediated action on asymmetric dimethylarginine (ADMA) and dimethylaminohydrolase (DDAH). Rat primary hepatic stellate cells (HSCs) were treated with TGF-β1 (5.0 ng/ml) alone or in combination with increasing concentrations of p38 MAPK specific inhibitor SB203580 (0, 1, 2 and 3 μM). Protein levels of DDAH1 and ADMA, and the activity of DDAH were assayed as described in Materials and Methods. (A) Dose-dependent effect of p38 MAPK specific inhibitor SB203580 on TGF-β1-mediated action on the DDAH1 protein expression, and (B) the DDAH1 activity; (C) Dose-dependent inhibitory effect of p38 MAPK specific inhibitor SB203580 on TGF-β1-mediated action on ADMA levels in HSCs culture medium. *P<0.05, **P<0.01 vs. control.

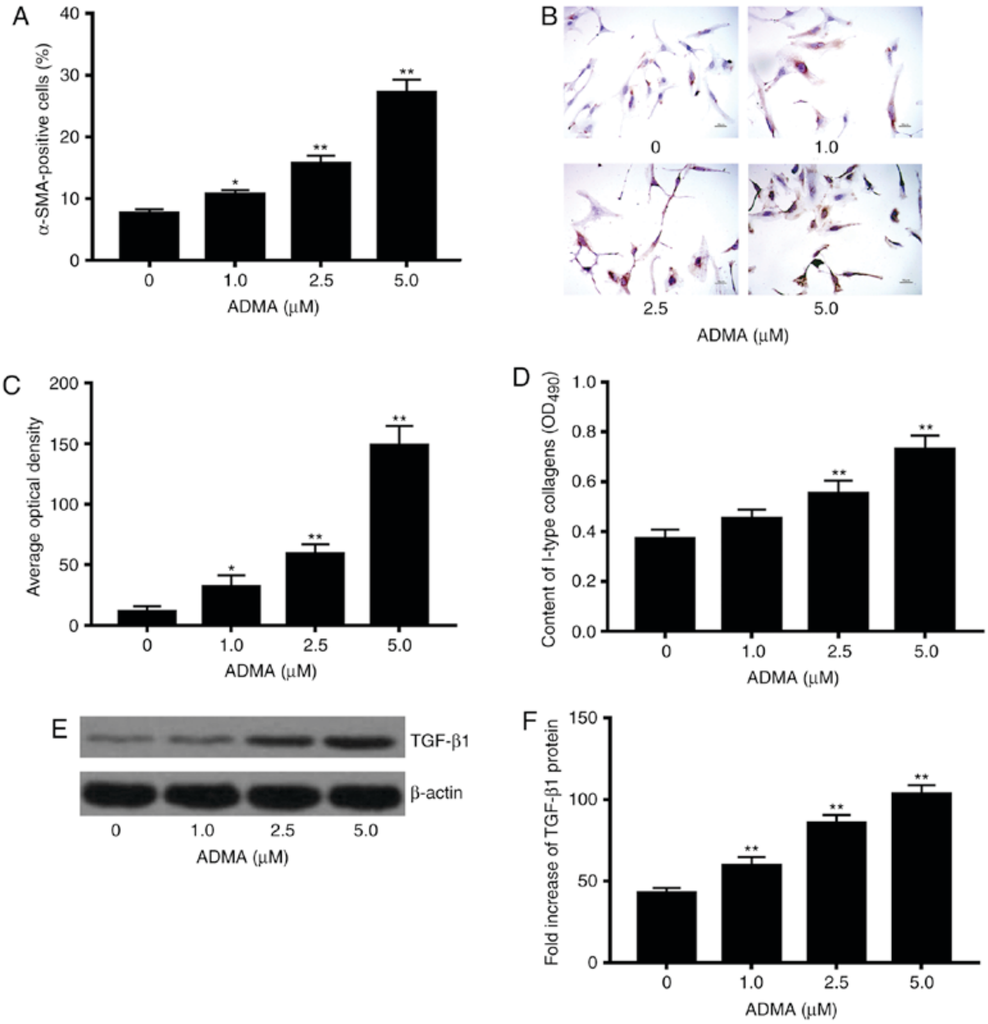

Figure 4. Effects of asymmetric dimethylarginine (ADMA) treatment on α-SMA-positive hepatic stellate cells (HSCs), intensity of α-SMA, and type I collagen in rat primary HSCs. Freshly prepared rat primary HSCs were treated with different concentrations of ADMA (0, 1, 2.5 and 5 μM) for 48 h. α-SMA-positive HSCs were assayed by IHC. α-SMA-positive HSCs were defined as those with light or dark brown particles visualized in the cell membrane and/or cytoplasm. The intensity of the α-SMA-positive HSCs was quantified with Image-ProPlus 6 digital medical image system. (A) Effect of ADMA on the number of the α-SMA-positive HSCs; (B) Representative images of IHC staining for α-SMA protein in the rat HSCs treated with increasing concentrations of ADMA; (C) effect of ADMA on α-SMA expression in rat HSCs. IHC staining intensity of the images as illustrated in (B) was quantified by calculating the average optical density (AOD) using Image-Pro Plus software 6.0 (Media Cybernetics, Silver Spring, MD, USA). ELISA for type I collagen was performed, and the concentrations of type I collagen were calculated by absorbance at 490 nm wavelength on a spectrophotometer, while western blot analysis was used to examine the protein levels of transforming growth factor (TGF)-β1 and β-actin; (D) effect of ADMA on levels of type I collagen in rat HSCs; (E) Effects of ADMA on protein expression of TGF-β1 in rat HSCs; (F) the bar graphs were generated from the above western blot analysis, showing the quantitative results after the amounts of TGF-β1 protein were normalized to those for β-actin, which did not vary with ADMA treatment. *P<0.05, **P<0.01 vs. control. Magnification, x200.

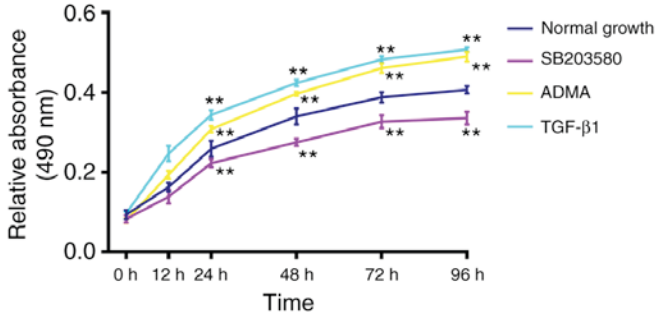

Figure 5. Effects of transforming growth factor (TGF)-β1, asymmetric dimethylarginine (ADMA) and SB203580 on proliferation of rat primary hepatic stellate cells (HSCs). Rat primary HSCs were treated with TGF-β1 (5.0 ng/ml), ADMA (5.0 μM), or SB203580 (3 μM) for 0, 12, 24, 48, 72, and 96 h. MTT assay was conducted to assess proliferation of HSCs and the results were expressed as absorbance at 490 nM. **P<0.01 vs. control.

by over-expression of DDAH diminished levels of TGF-beta 1, also well-documented a critical mediator in renal fibrogenesis, and that the tubuleinterstitial ischaemia was subsequently improved. Lluch *et al* (32) examined plasma concentrations of ADMA in patients with compensated alcohol cirrhosis and with advance cirrhosis in comparison with healthy controls, and found that the cirrhotic subjects had higher plasma levels of ADMA than the healthy subjects (ADMA ranged from 0.3 to 0.5 μmol/l). In this study, we initially performed a dose-dependent *in vitro* experiment by including lower concentrations of ADMA, and only at higher concentrations caused significant effects on the tested liver fibrotic markers, collagen type I (ADMA at concentrations of 2.5 and 5 μM) and α-SMA (ADMA at concentrations 1, 2.5, 5 μM). Based on this result, higher concentrations of ADMA were selected in this study, and we demonstrated that both α-SMA and type I collagen were markedly up-regulated in a dose-dependent fashion after exposure of freshly isolated HSCs to increasing concentrations of ADMA. These results were in agreement with the previous reports of the role of ADMA/DDAH in tissue fibrosis. It was worthy of more attention in our study that the ADMA-mediated action appeared to be achieved through the p38MAPK pathway as it was disrupted by the p38 MAPK specific inhibitor SB253080 but not by the inhibitors for the JNK- and ERK-MAPKs. In addition, the results obtained in this study supported the potentially novel mediator for the ADMA in the activation of HSCs and hepatic fibrosis. Studies have revealed a role of cytoskeleton actin in the ADMA-mediated activation of NF-κB and up-regulation of TGF-β in human renal glomerular endothelial cells (HRGECs) (29). Indeed, ADMA treatment stimulated assembly of stress fibers, induced DNA binding of NF-κB, and induced an increase in TGF-β1 expression in HRGECs. After cytoskeleton actin was disrupted, the activation of NF-κB was suppressed (29). ADMA has also been reported to increase TGF-β expression (33) and to induce kidney fibrosis (29) via activation of the NF-κB signaling pathway. However, the underlying molecular mechanisms were not elucidated.

Our study may have a number of limitations. First, the effective concentrations of TGF-β1 used in this *in vitro* study were similar to those reported previously, but much higher than the plasma levels of TGF-β1 observed in patients with liver fibrosis. Due to the short duration of exposure, lower concentration of TGF-β1 may fail to exert any effect on DDAH/ADMA, while long-term treatment in an experiment with freshly isolated HSCs seems to be challenging. Thus, a further study with long-term exposure to cytokine TGF-β1 in an *in vivo* animal model is required to clarify the effects of TGF-β1 on DDAH/ADMA and ADMA on the activation of HSCs. Second, the present study demonstrated that TGF-β1 decreases protein levels of DDAH, which may involve the p38MAPK signaling pathway, as disturbance of the p38 abrogates significantly the TGF-β1-mediated effects on DDAH/ADMA, while we could not exclude the possibility that the effect of TGF-β1 on DDAH/ADMA could be indirect. These need to be clarified by further investigations in future. Further studies are underway in our laboratory in this direction.

In summary, our findings on the effects of TGF-β on DDAH/ADMA, along with those reported in previous studies, suggest an importance of the interaction of TGF-β1 and DDAH/ADMA pathways in inducing HSCs activation, and that suppression of the ADMA/DDAH1 pathway could be an alterative approach to combating liver fibrosis.

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References

1. Koyama Y, Xu J, Liu X and Brenner DA: New developments on the treatment of liver fibrosis. Dig Dis 34: 589-596, 2016.
2. Lee YA, Wallace MC and Friedman SL: Pathobiology of liver fibrosis: A translational success story. Gut 64: 830-841, 2015.
3. Zhou WC, Zhang QB and Qiao L: Pathogenesis of liver cirrhosis. World J Gastroenterol 20: 7312-7324, 2014.
4. Li J, Wang W and Shen JL: The role of TGFbeta1 and IL-13 in cellular signal transduction of hepatic fibrosis of schistosomiasi. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 27: 357-360, 2009 (In Chinese).
5. Fabregat I, Moreno-Càceres J, Sánchez A, Dooley S, Dewidar B, Giannelli G and Ten Dijke P; IT-LIVER Consortium: TGF-β signalling and liver disease. FEBS J 283: 2219-2232, 2016.
6. Dooley S and ten Dijke P: TGF-β in progression of liver disease. Cell Tissue Res 347: 245-256, 2012.
7. Sakai K, Jawaid S, Sasaki T, Bou-Gharios G and Sakai T: Transforming growth factor-β-independent role of connective tissue growth factor in the development of liver fibrosis. Am J Pathol 184: 2611-2617, 2014.
8. Hayashi H and Sakai T: Biological significance of local TGF-β activation in liver diseases. Front Physiol 3: 12, 2012.
9. Massague J: TGFβ signalling in context. Nat Rev Mol Cell Biol 13: 616-630, 2012.
10. Dayoub H, Achan V, Adimoolam S, Jacobi J, Stuehlinger MC, Wang BY, Tsao PS, Kimoto M, Vallance P, Patterson AJ and Cooke JP: Dimethylarginine dimethylaminohydrolase regulates nitric oxide synthesis: Genetic and physiological evidence. Circulation 108: 3042-3047, 2003.
11. Arrigoni F, Ahmetaj B and Leiper J: The biology and therapeutic potential of the DDAH/ADMA pathway. Curr Pharm Des 16: 4089-4102, 2010.
12. Matsumoto Y, Ueda S, Yamagishi S, Matsuguma K, Shibata R, Fukami K, Matsuoka H, Imaizumi T and Okuda S: Dimethylarginine dimethylaminohydrolase prevents progression of renal dysfunction by inhibiting loss of peritubular capillaries and tubulointerstitial fibrosis in a rat model of chronic kidney disease. J Am Soc Nephrol 18: 1525-1533, 2007.
13. Ferrigno A, Rizzo V, Bianchi A, Di Pasqua LG, Berardo C, Richelmi P and Vairetti M: Changes in ADMA/DDAH pathway after hepatic ischemia/reperfusion injury in rats: the role of bile. Biomed Res Int 2014: 627434, 2014.
14. Baranyi A, Meinitzer A, Putz-Bankuti C, Stauber R, Kapfhammer HP and Rothenhäusler HB: Asymmetric dimethylarginine responses during interferon-α-induced depression in patients with chronic hepatitis C infection. Psychosom Med 76: 197-207, 2014.
15. Mookerjee RP, Malaki M, Davies NA, Hodges SJ, Dalton RN, Turner C, Sen S, Williams R, Leiper J, Vallance P and Jalan R: Increasing dimethylarginine levels are associated with adverse clinical outcome in severe alcoholic hepatitis. Hepatology 45: 62-71, 2007.
16. Sharma JN, Al-Omran A and Parvathy SS: Role of nitric oxide in inflammatory diseases. Inflammopharmacology 15: 252-259, 2007.
17. Kelly LK, Wedgwood S, Steinhorn RH and Black SM: Nitric oxide decreases endothelin-1 secretion through the activation of soluble guanylate cyclase. Am J Physiol Lung Cell Mol Physiol 286: L984-L991, 2004.
18. Failli P, DeFRANCO RM, Caligiuri A, Gentilini A, Romanelli RG, Marra F, Batignani G, Guerra CT, Laffi G, Gentilini P and Pinzani M: Nitrovasodilators inhibit platelet-derived growth factor-induced proliferation and migration of activated human hepatic stellate cells. Gastroenterology 119: 479-492, 2000.
19. Knook DL, Seffelaar AM and de Leeuw AM: Fat-storing cells of the rat liver. Their isolation and purification. Exp Cell Res 139: 468-471, 1982.
20. Shu JC, Zhao JR, Yang DH, Shen Y and Zhong CC: An improved method for the isolation of rat hepatic stellate cells. Zhonghua Gan Zang Bing Za Zhi 12: 353-355, 2004 (In Chinese).
21. Chang W, Yang M, Song L, Shen K, Wang H, Gao X, Li M, Niu W and Qin X: Isolation and culture of hepatic stellate cells from mouse liver. Acta Biochim Biophys Sin (Shanghai) 46: 291-298, 2014.
22. Jiang DJ, Jiang JL, Tan GS, Du YH, Xu KP and Li YJ: Protective effects of daviditin A against endothelial damage induced by lysophosphatidylcholine. Naunyn Schmiedebergs Arch Pharmacol 367: 600-606, 2003.

23. Kasumov T, Edmison JM, Dasarathy S, Bennett C, Lopez R and Kalhan SC: Plasma levels of asymmetric dimethylarginine in patients with biopsy-proven nonalcoholic fatty liver disease. Metabolism 60: 776-781, 2011.
24. Ferrigno A, Di Pasqua LG, Berardo C, Richelmi P and Vairetti M: Liver plays a central role in asymmetric dimethylarginine-mediated organ injury. World J Gastroenterol 21: 5131-5137, 2015.
25. Lluch P, Segarra G and Medina P: Asymmetric dimethylarginine as a mediator of vascular dysfunction in cirrhosis. World J Gastroenterol 21: 9466-9475, 2015.
26. Wang W, Zhao C, Zhou J, Zhen Z, Wang Y and Shen C: Simvastatin ameliorates liver fibrosis via mediating nitric oxide synthase in rats with non-alcoholic steatohepatitis-related liver fibrosis. PLoS One 8: e76538, 2013.
27. Sharma V, Ten Have GA, Ytrebo L, Sen S, Rose CF, Dalton RN, Turner C, Revhaug A, van-Eijk HM, Deutz NE, *et al*: Nitric oxide and L-arginine metabolism in a devascularized porcine model of acute liver failure. Am J Physiol Gastrointest Liver Physiol 303: G435-G441, 2012.
28. Li JC, Chang L, Lu D, Jiang DJ and Tan DM: Effect of asymmetric dimethylarginine on the activation of hepatic stellate cells and its mechanism. Zhong Nan Da Xue Xue Bao Yi Xue Ban 32: 427-432, 2007 (In Chinese).
29. Wang L, Zhang D, Zheng J, Feng Y, Zhang Y and Liu W: Actin cytoskeleton-dependent pathways for ADMA-induced NF-κB activation and TGF-β high expression in human renal glomerular endothelial cells. Acta Biochim Biophys Sin (Shanghai) 44: 918-923, 2012.
30. Mihout F, Shweke N, Bigé N, Jouanneau C, Dussaule JC, Ronco P, Chatziantoniou C and Boffa JJ: Asymmetric dimethylarginine (ADMA) induces chronic kidney disease through a mechanism involving collagen and TGF-β1 synthesis. J Pathol 223: 37-45, 2011.
31. Shibata R, Ueda S, Yamagishi S, Kaida Y, Matsumoto Y, Fukami K, Hayashida A, Matsuoka H, Kato S, Kimoto M and Okuda S: Involvement of asymmetric dimethylarginine (ADMA) in tubulointerstitial ischaemia in the early phase of diabetic nephropathy. Nephrol Dial Transplant 24: 1162-1169, 2009.
32. Lluch P, Torondel B, Medina P, Segarra G, Del Olmo JA, Serra MA and Rodrigo JM: Plasma concentrations of nitric oxide and asymmetric dimethylarginine in human alcoholic cirrhosis. J Hepatol 41: 55-59, 2004.
33. Feng Y, Zhang D, Zhang Y, Zhang Q and Liu W: The mechanism of long-term low-dose asymmetric dimethylarginine inducing transforming growth factor-β expression in endothelial cells. Int J Mol Med 31: 67-74, 2013.